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GENETIC AND MOLECULAR STUDIES OF THE PHLEBOTOMUS FEVER
GROUP OF VIRUSES

ANNUAL REPORT

DR. DAVID H.L. BISHOP
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In the current reporting period we have made the following progress relevant to the aims of the contract. We have: (1) characterized at the biochemical level the major viral induced **intracellular** antigens of Karimabad (KAR), Aguagate (AGU), Naples Sandfly Fever (SFN), Candiru (CDU), Buenaventura (BUE) and Sicilian Sandfly Fever (SFS); we have also characterized alternate isolates of Punta Toro (PT) virus that have different virulence markers (the viruses were originally obtained from various parts of Panama and were made available through the courtesy of USAMRIID personnel); (2) obtained reassortant PT viruses between prototype PT virus and an alternate PT strain; (3) used such reassortants to demonstrate that the phlebovirus S RNA codes for the viral N polypeptide and, from biochemical studies, shown that the S RNA also codes for a viral induced nonstructural polypeptide (NS_c); (4) analyzed the reassortment virus capabilities of PT, Icoraci (ICO) and BUE phleboviruses; (5) developed a convenient assay for demonstrating phleboviral interactions that may be useful in screening for genetically compatible phleboviruses (the procedure involves assays of the interference capabilities using ICO and PT DI virus stocks); (6) characterized DI preparations of ICO and PT virus stocks, demonstrating that they have new RNA species representing deleted L RNA segments; and (7) characterized the 3' end RNA sequences of several phleboviruses. The results of the current reported period are therefore as follows:

(1). The viral induced polypeptides, (immune precipitated from infected cell extracts by their homologous antisera), have been characterized for KAR, AGU, BUE, CDU, SFS and SFN viruses. Several alternate isolates of PT virus obtained from the eastern, central and western regions of Panama have been cloned and their L, M and S RNA species fingerprinted and shown to be distinct/related to each other (depending on the virus isolate).

(2). By dual wild-type virus crosses, reassortant viruses have been obtained between prototype PT and an alternate PT isolate (PT-ada). The genotypes of 2 such reassortants were shown by fingerprint analyses to be the L/M/S combinations of ada/PT/ada and PT/PT/ada.

(3). The N polypeptides of PT-ada and prototype PT viruses can be distinguished by tryptic peptide analyses allowing the N polypeptides of the reassortants to be analyzed. By such analyses it has been shown that their S RNA codes for the virion N polypeptide. From S mRNA in vitro translation analyses, this conclusion has been confirmed, with results that also indicate that the S mRNA directs the synthesis of a non-structural polypeptide (NS_S) that appears to be unrelated by primary sequence to the viral N polypeptide.

(4). Dual wild-type virus infections have failed to detect reassortant virus formation between PT and BUE viruses, or BUE and ICO viruses.

(5). An assay of the homologous and heterologous virus interference capabilities using by stocks of PT, or ICO, viruses that contain their respective defective interfering (DI) virus has been developed. Using such assays, homologous virus interference has been demonstrated. No heterologous virus interference has been detected from ICO DI and PT coinfections, or ICO DI and BUE, or ICO DI and snowshoe hare (SSH) bunyavirus coinfections.

(6). The stocks of PT and ICO DI virus have been shown to contain new RNA species not found in virus stocks lacking DI virus. Fingerprint analyses have shown that for both viruses the new RNA species represent deletion derivatives of their respective L RNA species.

(7). The 3' end sequence of the 3 RNA species of both PT and BUE viruses have been shown to be like those of the uukuvirus, Uukuniemi, i.e., $_{HO}$UUC.

I. SUMMARY

The reporting period represents the last 12 of the 42 months since the inception of the project. During the previous reporting periods we have: (1) characterized the major structural components of several Phlebotomus fever group viruses (Robeson, et al, 1979; Bishop et al., 1980: Cash et al., 1981), (2) isolated temperature sensitive (ts), conditional lethal, mutants of Punta Toro (PT) virus and categorized them by complementation-recombination analyses, (3) demonstrated the feasibility of using oligonucleotide fingerprinting to distinguish both Dengue (DEN) virus serotypes using cloned Aedes albopictus cells to produce labeled virus (Vezza et al., 1980) and Rift Valley fever (RVF) virus isolates obtained from various parts of Africa (Uganda, Rhodesia and South Africa, including the recent isolates from Egypt; Cash et al., 1981), (4) undertaken electron microscopic analyses on various phleboviruses; (5) analyzed, by radioimmune assays (RIA), the antigenic relationships of Karimabad (KAR), Chagres (CHG) and sandfly fever virus, Sicilian, (SFS) glycoproteins (G1, G2) and nucleocapsid (N)proteins (Klimas et al., 1981), and (6) initiated genetic analyses on Icoraci (ICO) phlebovirus. A synopsis of the results of the prior reporting periods is presented below:

(1). Analyses of the major structural components of the Phlebotomus fever viruses established that KAR, PT, CHG, Candiru (CDU), ICO, PHL 3, Itaporanga (ITP), Buenaventura (BUE), and the Sicilian and Naples sandfly fever (SFS, SFN) viruses, each has a tripartite RNA genome and three major structural polypeptides (two external glycoproteins, G1 and G2, $57-69 \times 10^3$ daltons, and an internal, nucleocapsid associated, protein N, $20-24 \times 10^3$ daltons). Both the mol. wt. of the major structural polypeptides and the virion RNA segments of the different Phlebotomus fever viruses can be easily distinguished from those of bunyaviruses (e.g. the California serogroup, CAL, viruses) and, to various extents (depending on the virus), from each other. Tryptic peptide analyses of ^{35}S and ^{3}H methionine labeled G1 and G2 polypeptides of KAR have established that these two polypeptides have distinguishable sequences. The behaviour of reduced KAR G1 and G2 polypeptides on polyacrylamide gel electrophoresis was found to be aberrant by comparison with unreduced preparations.

(2). Twenty four ts mutants of PT virus were isolated following mutagenesis of the wild-type virus by growth in the presence of 5-fluorouracil. Recombination assays with these mutants have allowed them to be categorized into 3 non-overlapping recombination groups (Group I has 8 ts mutants; Group II has 5 ts mutants; Group III has 1 ts mutant - so strictly is not a group - and 1 ts mutant is probably a double mutant).

(3). Oligonucleotide fingerprint analyses of prototype DEN 1, 2, 3 and 4 viruses involving both single and mixed coelectropherograms of ribonuclease T1 digests of ^{32}P labeled 40S viral RNA samples showed that each has a unique fingerprint that is easily distinguished from that of another prototype DEN virus (these procedures have now been adopted by WRAIR personnel for their Dengue virus studies). Evidence was obtained indicating that the 5' sequence of DEN 2 RNA is m7GpppAmpXp...

1

(4). Electron microscopic analyses of the surface structure arrangments of PHL group viruses have been undertaken and indicate that unlike bunyaviruses, but like uukuviruses (2 of the other Bunyaviridae genera) PHL group viruses have a particular "chimney-pot" arrangements of their surface glycoproteins as evidenced by glutaraldehyde fixation prior to staining.

(5). Competition RIA assays using iodinated nucleocapsid and glycoprotein preparations of KAR virus, KAR antisera and the competing antigens of KAR, CHG and SFS indicate that the KAR and SFS N polypeptides have more antigenic determinants in common than have the N polypeptides of KAR and CHG. Also the KAR and SFS G polypeptides share more antigenic determinants than the G polypeptides of KAR and CHG. No shared antigenic determinants were detected between KAR and vesicular stomatitis virus (VSV), or the bunyaviruses La Crosse (LAC), Oriboca (ORI), or Bunyamwera (BUN) viruses.

(6). ICO virus has been adapted to produce plaques in Vero cell monolayers at $39.8^{\circ}C$. The original virus stock, which gave 10^5 plaques at $35^{\circ}C$, gave none at $39.8^{\circ}C$. By high temperature passaging of the virus stock and cloning at $39.8^{\circ}C$, a stock of ICO virus has been derived which gives $8x10^4$ PFU at $35^{\circ}C$ and $2.3x10^3$ PFU at $39.8^{\circ}C$.

In the current reporting period we have made the following progress relevant to the aims of the contract. We have: (7) characterized at the biochemical level the major viral induced intracellular antigens of KAR, Aguagate (AGU), SFN, CDU, BUE and SFS; we have also characterized alternate isolates of PT virus that have different virulence markers (the viruses were originally obtained from various parts of Panama and were made available through the courtesy of USAMRIID personnel); (8) obtained reassortant PT viruses between prototype PT virus and an alternate PT strain; (9) used such reassortants to demonstrate that the phlebovirus S RNA codes for the viral N polypeptide and, from biochemical studies, shown that the S RNA also codes for a viral induced nonstructural polypeptide (NS_s); (10) analyzed the reassortment virus capabilities of PT, ICO and BUE phleboviruses; (11) developed a convenient assay for demonstrating phleboviral interactions that may be useful in screening for genetically compatible phleboviruses (the procedure involves assays of the interference capabilities using ICO and PT DI virus stocks); (12) characterized DI preparations of ICO and PT virus stocks, demonstrating that they have new RNA species representing deleted L RNA segments; and (13) characterized the 3' end RNA sequences of several phleboviruses. The results of the current reported period are therefore as follows:

(7). The viral induced polypeptides, (immune precipitated from infected cell extracts by their homologous antisera), have been characterized for KAR, AGU, BUE, CDU, SFS and SFN viruses. Several alternate isolates of PT virus obtained from the eastern, central and western regions of Panama have been cloned and their L, M and S RNA species fingerprinted and shown to be distinct/related to each other (depending on the virus isolate).

(8). By dual wild-type virus crosses, reassortant viruses have been obtained between prototype PT and an alternate PT isolate (PT-ada). The genotypes of 2

2

such reassortants were shown by fingerprint analyses to be the L/M/S combinations of ada/PT/ada and PT/PT/ada.

(9). The N polypeptides of PT-ada and prototype PT viruses can be distinguished by tryptic peptide analyses allowing the N polypeptides of the reassortants to be analyzed. By such analyses it has been shown that their S RNA codes for the virion N polypeptide. From S mRNA in vitro translation analyses, this conclusion has been confirmed, with results that also indicate that the S mRNA directs the synthesis of a non-structural polypeptide (NS_S) that appears to be unrelated by primary sequence to the viral N polypeptide.

(10). Dual wild-type virus infections have failed to detect reassortant virus formation between PT and BUE viruses, or BUE and ICO viruses.

(11). An assay of the homologous and heterologous virus interference capabilities using by stocks of PT, or ICO, viruses that contain their respective defective interfering (DI) virus has been developed. Using such assays, homologous virus interference has been demonstrated. No heterologous virus interference has been detected from ICO DI and PT coinfections, or ICO DI and BUE, or ICO DI and snowshoe hare (SSH) bunyavirus coinfections.

(12). The stocks of PT and ICO DI virus have been shown to contain new RNA species not found in virus stocks lacking DI virus. Fingerprint analyses have shown that for both viruses the new RNA species represent deletion derivatives of their respective L RNA species.

(13). The 3' end sequence of the 3 RNA species of both PT and BUE viruses have been shown to be like those of the uukuvirus, Uukuniemi, i.e., $_{HO}$UUC.

II.   REPORT

A.   Introduction.

The objectives of this contract are to analyse the genetic capacity of members of the Phlebotomus fever (PHL) group viruses (Phlebovirus genus, Bunyaviridae) and determine the implications of forming new Phlebotomus fever virus genotypes by genome segment reassortment. We have shown that the member viruses of this genus of the family Bunyaviridae have unique tripartite RNA genomes (with RNA species designated L, M and S respectively) and are capable (in dual PT ts mutant virus infections) of reassorting their RNA segments to produce wild-type viruses. Since members of this group of exotic viruses cause illnesses in epidemic proportions (e.g., RVF) in different parts of the world, and therefore are of military significance, our objectives relate to the question of deriving vaccine strains of viruses which will be useful in protecting military and civilian personnel against virus infections.

To realize these objectives, part of our research has been to determine which PHL group viruses are genetically compatible (capable of producing reassortant viruses with other members of the genus, or members of other genera of the family). Studies conducted in our laboratories have demonstrated that certain distinct virus serotypes within the CAL serogroup of the Bunyavirus genus (another genus of the Bunyaviridae) are capable of RNA segment reassortment inter se (Gentsch & Bishop, 1976; Gentsch, et al., 1977b; Gentsch et al., 1979; Rozhon et al., 1981). However, not all CAL bunyavirus crosses yeild reassortants, indicating that their gene pool is limited. So for no crosses between viruses representing different bunyavirus serogroups (e.g., CAL and members of the Bunyamwera serogroups) have yielded reassortant viruses. Results reported in this current reporting period likewise document the inability of certain PHL group viruses to produce reassortant viruses. However, studies with PT virus varieties show that reassortant viruses can be obtained between related phleboviruses. Thus it appears that, as in the case of bunyaviruses, there are restrictions in genetic interactions among PHL group viruses. Defining these restrictions and determining the available gene pools for particular PHL group viruses are the goals of our continued studies.

By knowing the RNA segment coding assignments (see Gentsch & Bishop, 1978, 1979), we propose to produce custom genotypes of particular genetically compatible Phlebotomus fever group viruses containing genetic information and gene products derived from both parental viruses. Certain of our studies are therefore directed towards defining the RNA segment coding assignments of phleboviruses. New reassortant virus genotypes will eventually be tested for their pathogenicity and vaccine capabilities in model animal systems and compared to the prototype strains. If effective, then similar procedures will be used to derive reassortant viruses in clean cell systems in order to obtain virus vaccines that can be used to immunize man.

This report therefore describes both analyses of the genetic potential of selected PHL group viruses, and molecular studies to characterize the viral genomic RNA species and their gene products. The report covers items 7-13 listed in the Summary. It will not detail the results given in previous reports.

7) Biochemical analyses of PHL group viruses, including alternate isolates of PT virus.

a. RNA analyses.

The RNA sizes and distinguishable L, M and S RNA fingeprint information developed in prior years for KAR, PT, ITP, CDU, CHG, SFS, SFN, BUE, and ICO phleboviruses has been extended to include 5 alternate isolates of PT virus. The new PT isolates (and their origins) are as follows: PT Bayano (1975, eastern Panama, Lutzomyia sanguinaria sandflies), PT Bayano (1976, also eastern Panama, sentinel hamster), PT Adamas (PT-ada, 1974, about 100 miles further east in Panama than the Bayano isolates, human isolate), PT Aguacate (12/1970, western Panama, Lutzomyia trapidoi), and PT Aguacate (8/1970, western Panama, Lutzomyia spp.). Prototype PT virus was isolated from a febrile man in 1966 who had been in the Jungle Warfare Training Center, west region of the Canal Zone, central Panama. Two clones of prototype PT virus (B and T) of different passage histories have also been analyzed.

Interestingly, prototype PT and PT-ada (representing virus obtained from different geographic areas of Panama) exhibit different virulence properties in model animal systems (Dr. C.J. Peters, personal communication). Prototype PT is avirulent (up to a dose of 10^6 pfu) following ip inoculation in adult outbred hamsters whereas PT-ada kills such hamsters at or above doses equivalent to 10 pfu. These virulence differences will be useful markers to study the question of which genes code for phlebovirus virulence, as determined in analyses of intertypic reassortant PT viruses (see proposal).

4

For each alternate PT virus three RNA species (L,M,S) have been identified and from coelectropherograms of their viral RNA species with the viral RNA species of PT virus, appear to have equivalent molecular weights to those of the prototype strain. Composite and individual L, M and S RNA fingerprints (Clewley and Bishop, 1980; Robeson et al., 1979; Clewley et al., 1977a,b) have been obtained for the five new PT strains (Fig. 1-2). The results indicate that each virus has RNA species with distinguishable fingerprints and unique L, M and S RNA sequences. The 1975 and 1976 PT Bayano isolates are evidently closely related to each other. Their fingerprints differ from those of prototype PT, or the 3 other alternate PT isolates. Both strains of prototype PT virus give similar L,M and S fingerprints with at most only a few oligonucleotide differences (see Fig. 1,2).
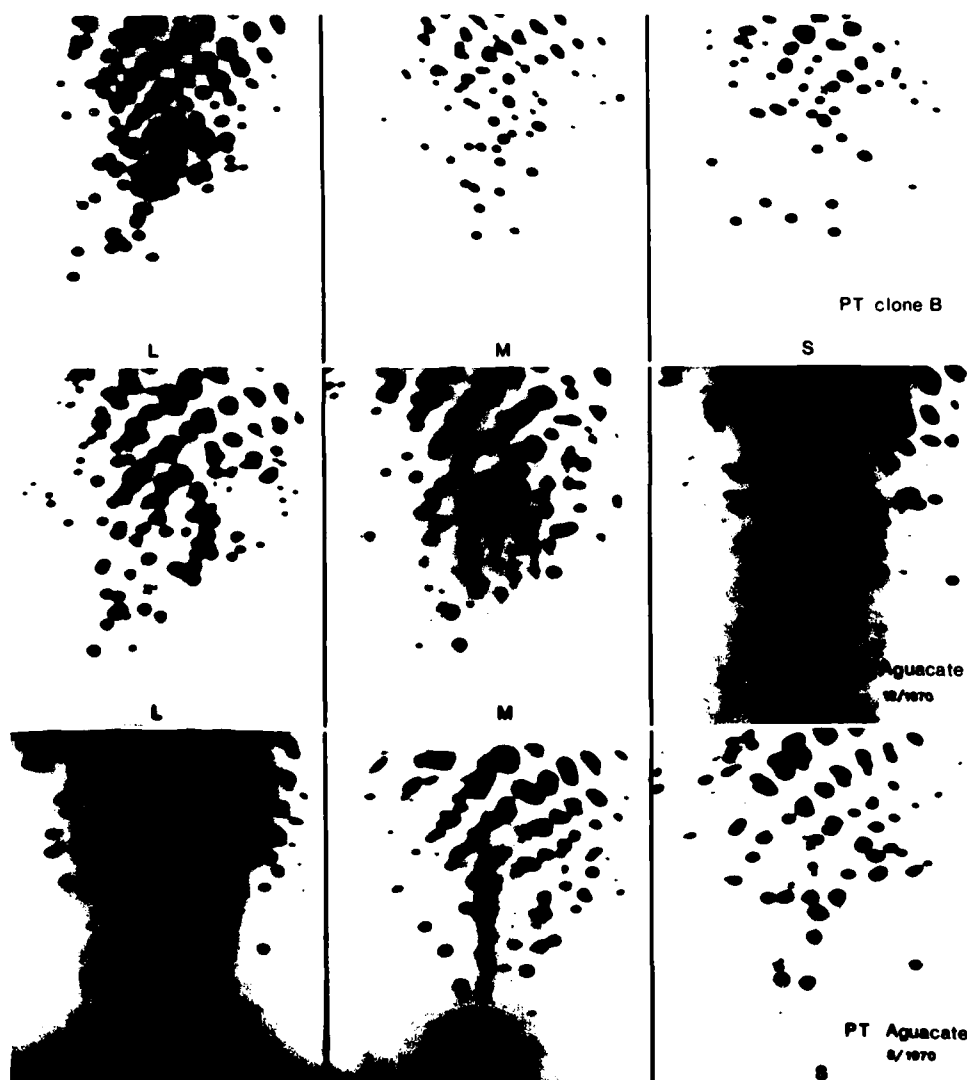

Figure 1

Individual L, M and S RNA fingerprints of prototype PT, clone B, PT Aguacate (12/19/70) and PT Aguacate (8/1970). The Xs represent the positions of reference dye markers.
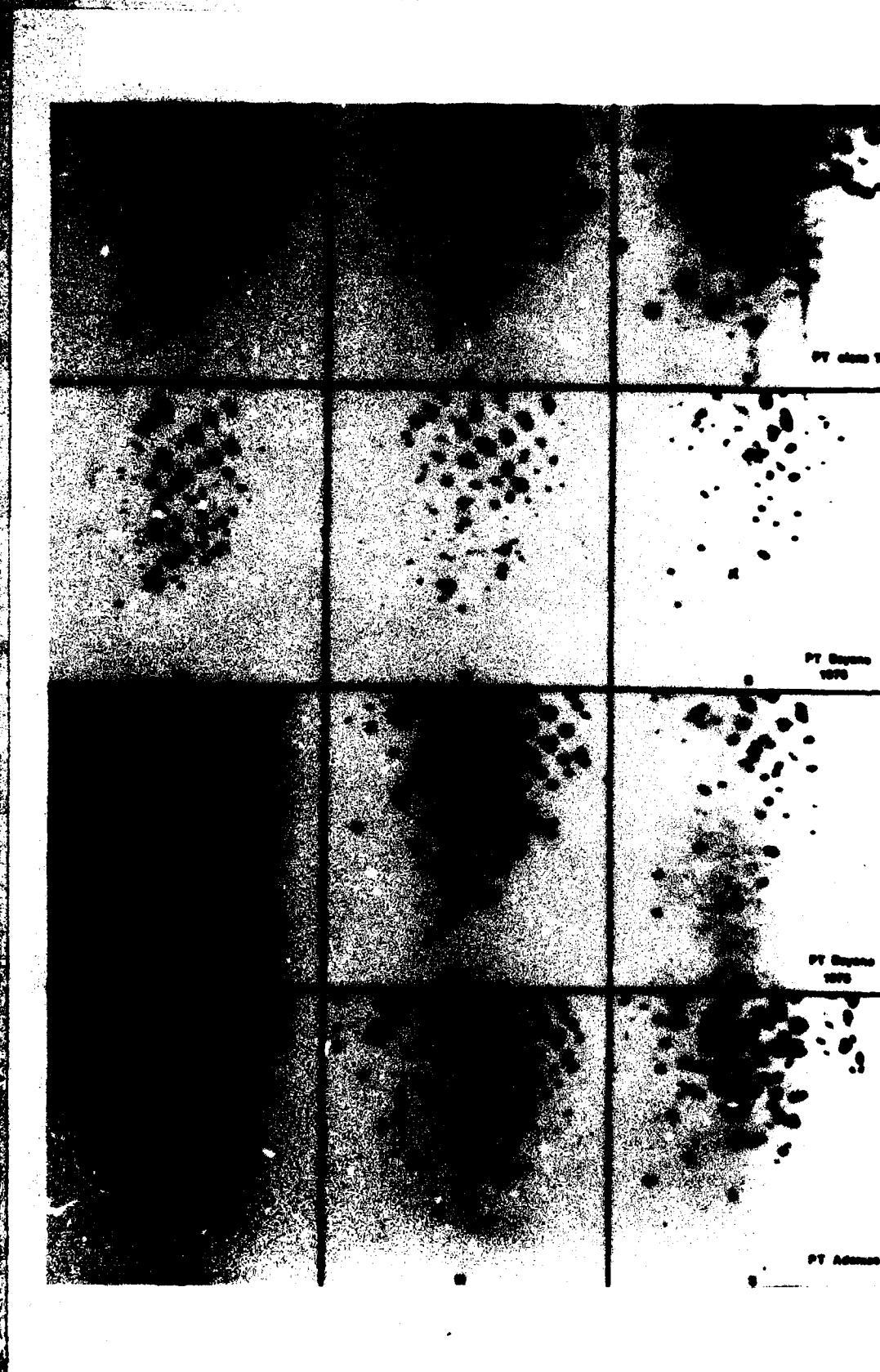
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Figure 2

RNA fingerprints of prototype PT, clone T, PT Bayano (1976),
PT Adames (PT-adm).

b. **Viral polypeptides.**

Previous studies with KAR, PT, CHG, CDU, ICO, PHL 3, ITP, SFS and SFN established that their major virion polypeptides include two glycoproteins (G1, G2) of size $58\text{-}69\text{x}10^3$ dalton plus a nucleocapsid polypeptide (N) of size $20\text{-}24\text{x}10^3$ daltons. In Fig. 3 are shown coelectropherograms of mixtures of immune precipitated infected cell extracts of (A) ^{35}S-methionine KAR + ^{3}H-methionine Aguagate (AGU), (B) KAR + BUE, (C) KAR + SFN, (D) KAR + CDU, (E) KAR + SFS (Sabin), and (F) KAR + SFS (Tesh).
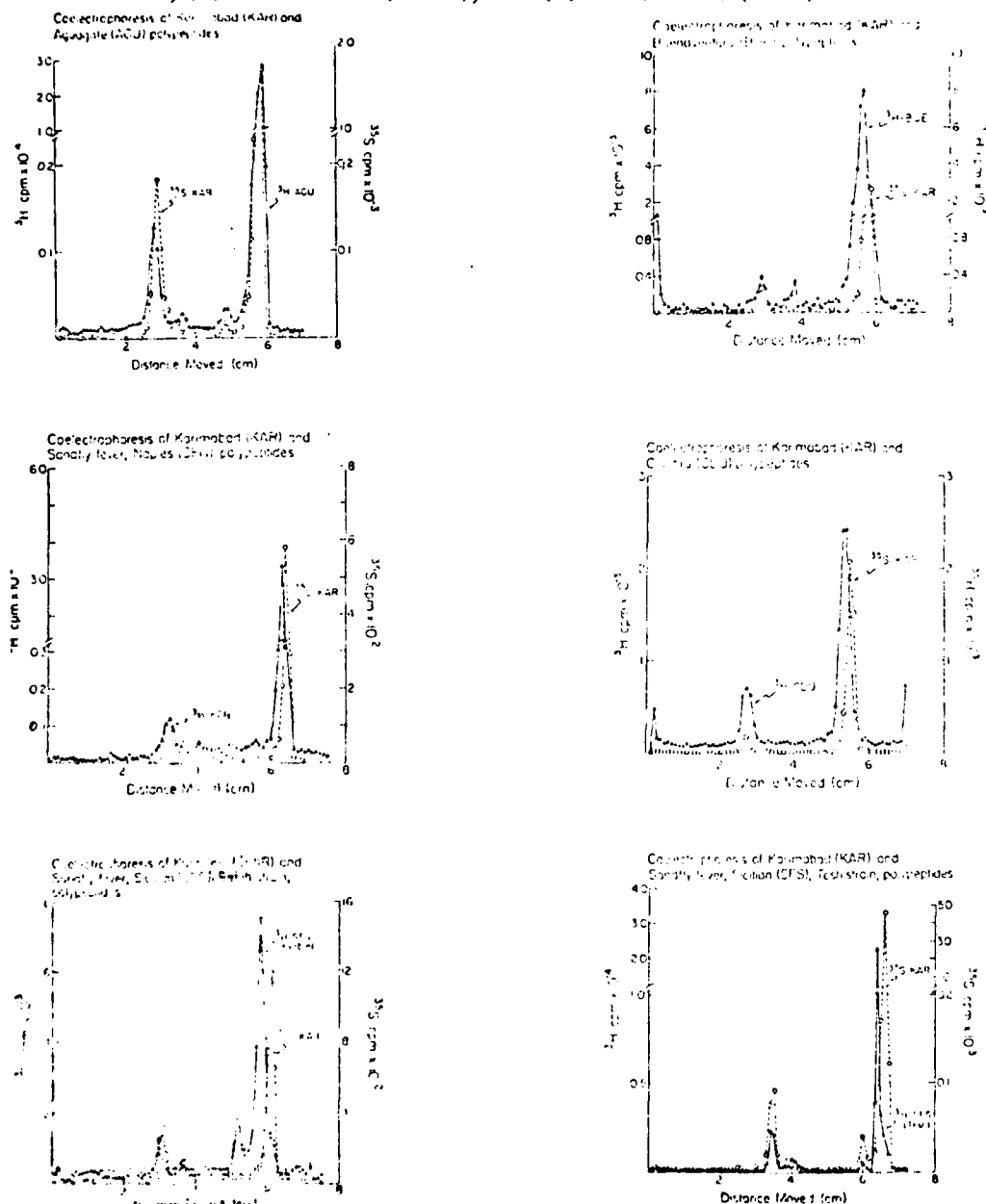

Figure 3

Coelectrophoresis of mixed immune precipitates of infected cell extracts of: (A) ^{35}S-methionine KAR and ^{3}H-methionine AGU, (B) ^{35}S-KAR + ^{3}H-BUE, (C) ^{35}S-KAR + ^{3}H-SFN, (D) ^{35}S-KAR + ^{3}H-CDU, (E) ^{35}S-KAR + ^{3}H-SFS (Sabin), (F) ^{35}S KAR + ^{3}H-SFS (Tesh).

As can be seen in Fig. 3, many of the PHL viruses induce an N polypeptide (recovered around 6 cm. in the gels). However, little intracellular glycoprotein is detected in the immune precipitates (3 cm region of the gels). Although not well immune precipitated for all samples, a 30×10^3 dalton polypeptide (p30) has been detected in many infected cell extracts, it may represent a nonstructural polypeptide induced in virus infected cells. It is not immune precipitated from mock infected cell extracts indicating that it is a viral induced polypeptide.

When ^{35}S-methionine labelled p30 and ^{3}H-methionine labelled KAR N polypeptides were recovered from the resolved immune precipitates of KAR virus infected cell extracts, digested with TPCK-trypsin and the resulting tryptic peptides resolved by ion exchange chromatography (Gentsch and Bishop, 1978) data shown in Fig. 4 were obtained.
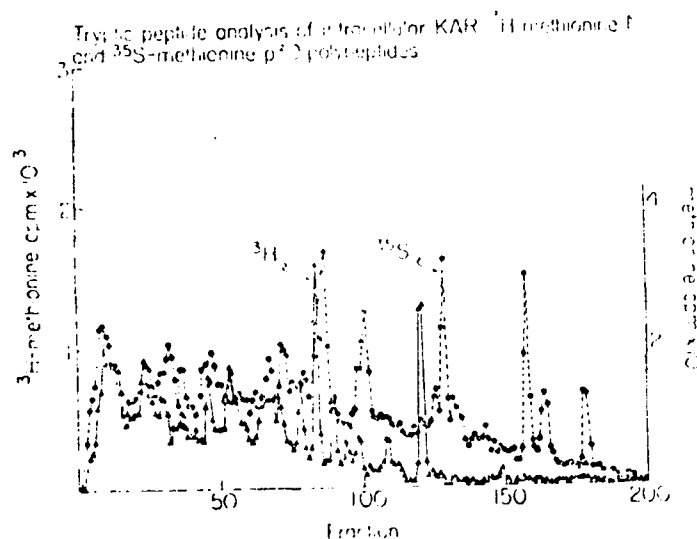

Figure 4

Tryptic peptide analyses of digests of ^{35}S-methionine p30 and ^{3}H-methionine N polypeptides recovered from immune precipitates of KAR virus infected cells.

The results suggest that the p30 polypeptide is not a precursor to the N polypeptide. The lack of several methionine labelled N tryptic peptides (e.g. those recovered between fractions 90 and 130) from the p30 pattern argue against p30 being a precursor to N. In parenthesis, pulse-chase analyses of KAR virus induced polypeptides have failed to demonstrate a precursor-product relationship between p30 and N. Thus we tentatively have concluded that KAR p30 and N are unrelated viral gene products (although they may be coded by the same viral RNA species).

8). <u>Genetic recombination between prototype PT and PT-Adamas (PT-ada).</u>

Rather than produce ts mutants of one of the alternate PT virus isolates and perform genetic recombination analyses beween the ts mutants of prototype PT virus and derived mutants of the alternate strain, we have elected to perform dual wild-type virus coinfections and seek reassortant viruses among randomly selected progeny virus clones. Another reason not to use ts mutants stems from our recent observations (in studies with bunyaviruses), that ts mutants often have silent, attenuating, mutations in RNA segments that do not have the change specifying the ts phenotype (Rozhon et al., 1981). Such attenuating mutations may be transferred to "wild-type" progeny of dual ts mutant virus crosses causing these progeny to be attenuated. Since one of the objects of our studies is to determine which viral gene products are the major determinants of virulence, obviously acquiring attenuating mutations would complicate our analyses.

Dual wild-type infections of Vero cells with prototype PT virus (MOI=10) and PT-ada (MOI=10) were performed under experimental conditions which should have yielded essentially equivalent numbers of both viruses by 36 hr post-infection. In fact, the yields of the dual virus infection (5×10^7/ml) were similar to the yields of parallel individual virus infections and represented about a 1000-fold increase over desorbed inocula viruses (i.e., those detected after washing the infected cells post-adsorption). Randomly selected clones of virus were recovered and their composite L+M+S RNA fingerprints obtained. Of 7 clones fingerprinted, 3 had L/M/S genotypes of PT/PT/PT, 2 were ada/ada/ada, 1 was PT/PT/ada and 1 was ada/PT/ada. The composite fingerprints of the recovered PT/PT/PT, ada/ada/ada and the two reassortants are shown in Fig. 5.
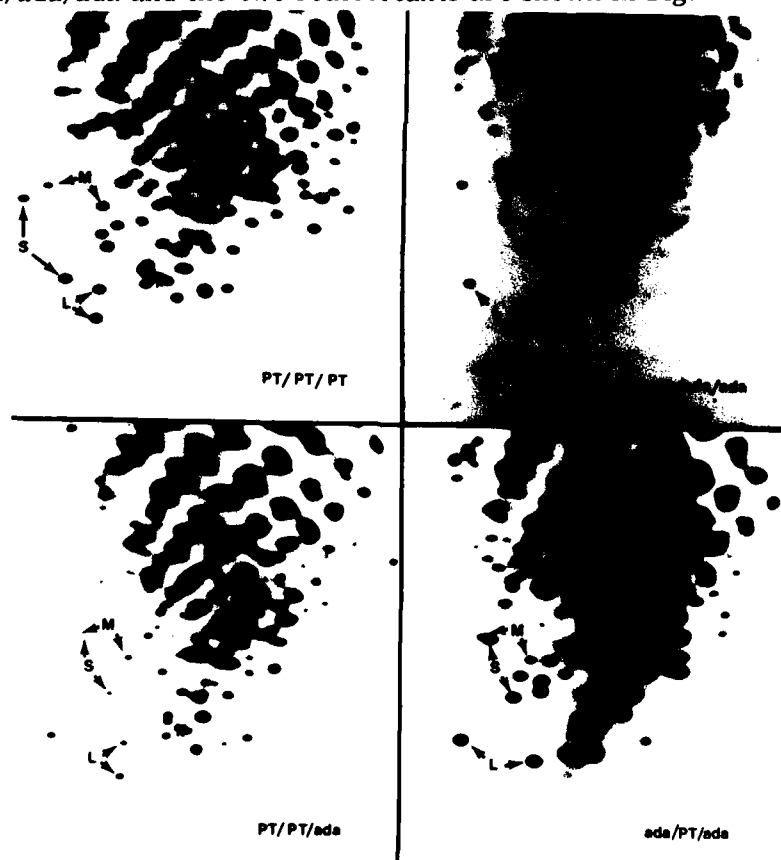

PT/PT/PT

ada/ada/ada

PT/PT/ada

ada/PT/ada

Figure 5

Composite L+M+S RNA fingerprints of prototype PT (PT/PT/PT), PT Adamas (ada/ada/ada), and the reassortants PT/PT/ada and ada/PT/ada. Unique PT and ada L, M and S oligonucleotides are indicated in each panel.

9

9). RNA coding assignments of phleboviruses.

Despite the fact that serologically PT-ada and prototype PT viruses are indistinguishable (Dr. C.J. Peters, personal communication), tryptic peptide analyses of their ^{3}H-lysine labeled viral N polypeptides allow them to be distinguished (Fig. 6, fractions 130-160). Although there is only a single lysine labelled tryptic peptide that differentiates these viruses, analyses of the tryptic peptide of the PT/PT/ada N polypeptide indicates that it corresponds to the ada N species rather than that of the prototype PT virus (Fig. 6). These results suggest therefore that the S RNA of these viruses codes for the N polypeptide.
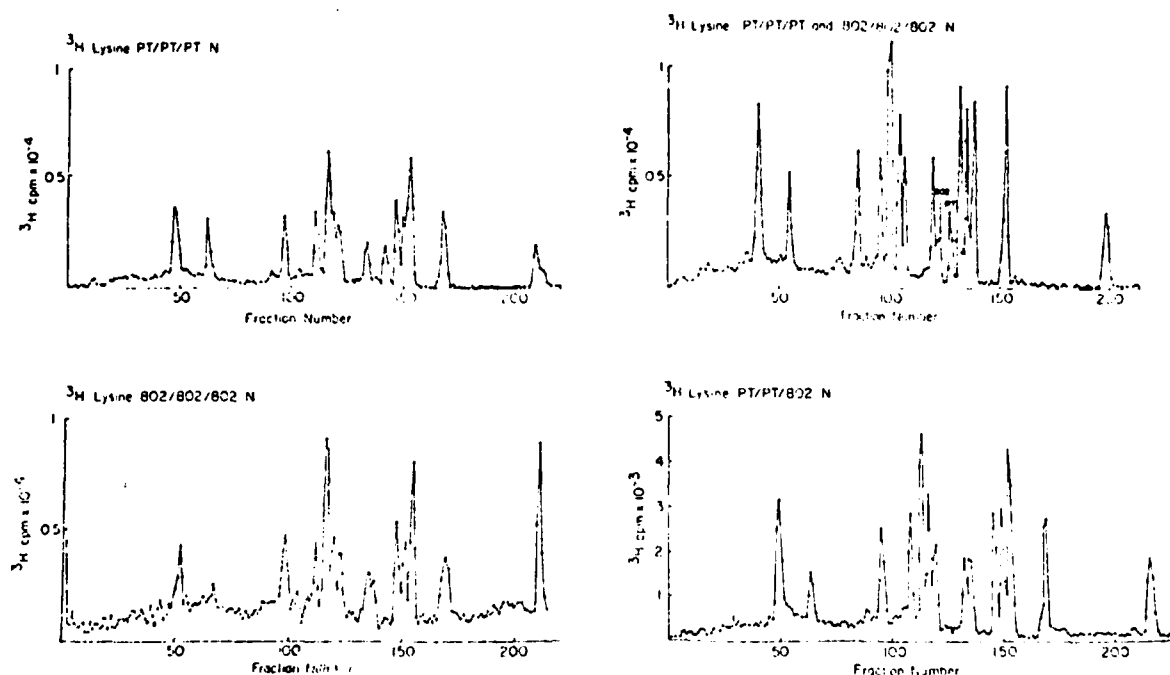

Figure 6

Tryptic peptide analyses of ^{3}H-lysine labelled prototype PT virus N polypeptide (PT/PT/PT, top left panel), ^{3}H-lysine labelled PT ada (802/802/802, bottom left panel), a mixture of ^{3}H-lysine labelled prototype PT and PT-ada N polypeptide (PT/PT/PT + 802/802/802, top right panel), and that of ^{3}H-lysine labelled PT/PT/ada N polypeptide (PT/PT/802, bottom right panel).

In preliminary analyses of KAR virus infected cell polysomal RNA extracts labelled between 5 and 10 hr post-infection (and after a 5 hr pretreatment with 1 ug/ml of Actinomycin D to reduce host cell RNA synthesis), four viral induced RNA species were identified. Three of these RNA species corresponded by size to virion RNA species. The fourth migrated faster than K^R S RNA, i.e., has a size of approximately $5x10^5$ daltons. After recovery of the fourth RNA from a preparative slab gel of low melting agarose, it was used to prime an in vitro rabbit reticulocyte lysate in the presence of high specific activity ^{3}H-leucine. The translation products were resolved by polyacrylamide gel electrophoresis. The results obtained indicate that this subgenomic RNA preparation directed the synthesis of both N and the p30 polypeptide. Hybrid arrest translation experiments using the S (but not with the M or L) vRNA species blocked translation of the N and p30 polypeptides. Tryptic peptide analyses of the products, and their comparison to those of the viral induced intracellular p30 and virion N polypeptides will be needed to confirm that p30 and N were made and are coded by the same (or related, e.g., sliced) viral mRNA species. If further analyses bear out this postulate, then the strategy of synthesis of the p30 (tentatively designated NS_S) and N polypeptides will be of interest to determine (independant translation initiation, polyprotein precursor, spliced mRNA species, etc).

10). <u>Genetic recombination between heterologous phleboviruses.</u>

We have employed two approaches to investigate the question of reassortment between different phlebovirus serotypes. In one approach we have used dual wild-type virus infections (PT x BUE; ICO x BUE) in a non-selective procedure to produce reassortants and in the other we have used PT <u>ts</u> mutants in coinfections with wild-type BUE virus (assaying the progeny at the non-permissive temperature) to screen for reassortants.

For both types of dual wild-type virus coinfections, in order to select potential reassortant progeny virus clones, we used the fact that the N polypeptides of the 2 parental virus serotypes can be readily differentiated. We reasoned that the principal criterion we needed to satisfy in a non-selective system was that the 2 viruses infect the same cells and in them grew equally well. This could be determined if we could show that for dual virus infections initiated at high MOI (e.g., an MOI of 10), the number of progeny virus was in excess of the desorbed virus inocula (1000 fold excess), and that there were essentially equivalent numbers of progeny clones that induced one type of N polypeptide in infected cells as those that induced the other. Since for ICO, BUE and PT viruses we are able to distinguish by slab polyacrylamide gel electrophoresis the sizes of both the viral N polypeptides (ICO: $20x10^3$, BUE: $21x10^3$, PT: $24x10^3$) and their glycoproteins (ICO: $62x10^3$, BUE: $58-60x10^3$, PT: $60-68x10^3$) - see last year's progress report - should evidence of segregation of the induced gene products in cloned progeny virus infected cell extracts be obtained, then we would be able to directly select for recombinant viruses. Once our objectives of essentially equivalent parental virus growth in cells infected at high multiplicity were realized, we were able to use oligonucleotide fingerprinting to determine if there were any reassortant virus genotypes among the cloned progeny.

From 16 cloned progeny obtained from the BUE x PT cross, we selected 8 which induced a BUE type N polypeptide (the amounts of G polypeptides detected in the infected cell extracts were too little to allow them to be classified as to their origin, see Fig. 3, and we did not want to purify virions for these assays). Fingerprint analyses demonstrated that all 8 had a genotype of BUE/BUE/BUE. Likewise of 8 cloned progeny that induced a PT type N polypeptide, all 8 were found to have genotypes of PT/PT/PT. Similar results were obtained for the cloned progeny of the ICO x BUE cross.

11

The second strategy we used to investigate the question of whether BUE and PT viruses could produce reassortments took advantage of the fact that wild-type PT virus produces plaques at both 35° and 39.8^O, whereas both our PT $\underline{ts}$ mutants and the wild-type BUE virus stock give plaques at 35°, but not at 39.8^O (see last year's report). Assuming that the inability of the BUE virus to produce plaques at 39.8^O is not a property of the gene products of each and every BUE RNA segment, we assayed for reassortant viruses that would grow at 39.8^O because they lacked the segment coding for the conditional lethal mutation of the PT $\underline{ts}$ mutant (i.e., ones that possessed a compensating BUE segment and its gene products).

The results of crosses of wild-type BUE and $\underline{ts}$ mutants of PT representing the Group I mutants ($\underline{ts}$ 1, $\underline{ts}$ 8), or Group II mutants ($\underline{ts}$ 2, $\underline{ts}$ $\overline{6}$), or Group III mutant ($\underline{ts}$ 18) indicated that no more wild-type progeny viruses were obtained from the mixed virus infections than from the single virus infections (i.e., viruses representing revertants or virus that leaked through the plaque assays; %R less than 0.01%).

Conducting recombination assays at 35°, or 39.8^O gave equivalent results. We cannot, of course, exclude the possibility that every BUE RNA segment codes for gene products that inhibit virus replication at 39.8^O, so that reassortants with PT RNA segments do not produce plaques at 39.8^O.

In conclusion, no evidence for recombination between PT and BUE, or ICO and BUE has been obtained.

11). The use of interference as a screening procedure for genetically compatible phleboviruses.

Although reassortant, intertypic, PT viruses have been obtained from dual infections involving prototype PT and an alternate PT isolate, (section 8), our inability to detect recombination between PT (or ICO) and BUE viruses suggests that the gene pool of these viruses may be limited. To define the PHL viruses that contribute to a common gene pool using the procedures outlined in the previous section would be a time consuming process. We have therefore sought for alternate methods to screen for genetically interactive phleboviruses.

One method we have recently investigated is that of heterologous virus interference. Although such interference does not guarantee that the viruses can also recombine, we have proceeded on the assumption that the lack of demonstrable interference probably shows that the viruses are not genetically compatible. On this assumption we have developed an interference assay for phleboviruses using stocks of ICO virus that, as will be shown, contain interfering virus. We also have stocks of PT virus that appear to contain interfering virus. Our goals are to screen for heterologous virus interference and if that can be demonstrated then test for recombination (see this year's proposal).

During our initial analyses of BUE virus, difficulties were experienced in obtaining high titered virus stocks (see last year's report). As virus stocks were passaged, their titers dropped. Such a phenomenon is typical of the presence and amplification of defective interfering (dI) virus (Huang & Baltimore, 1977). To investigate whether dI virus can be demonstrated in phlebovirus stocks, ICO virus was passaged undiluted in Vero cells. The virus titers of successive passage dropped from $2x10^8$ (2nd passage) to $9x10^5$ (5th passage). Virus yields were therefore determined in single and mixed virus infections involving the 2nd and 5th ICO virus stocks. The results, shown in Fig. 7, clearly demonstrated homologous ICO virus interference.
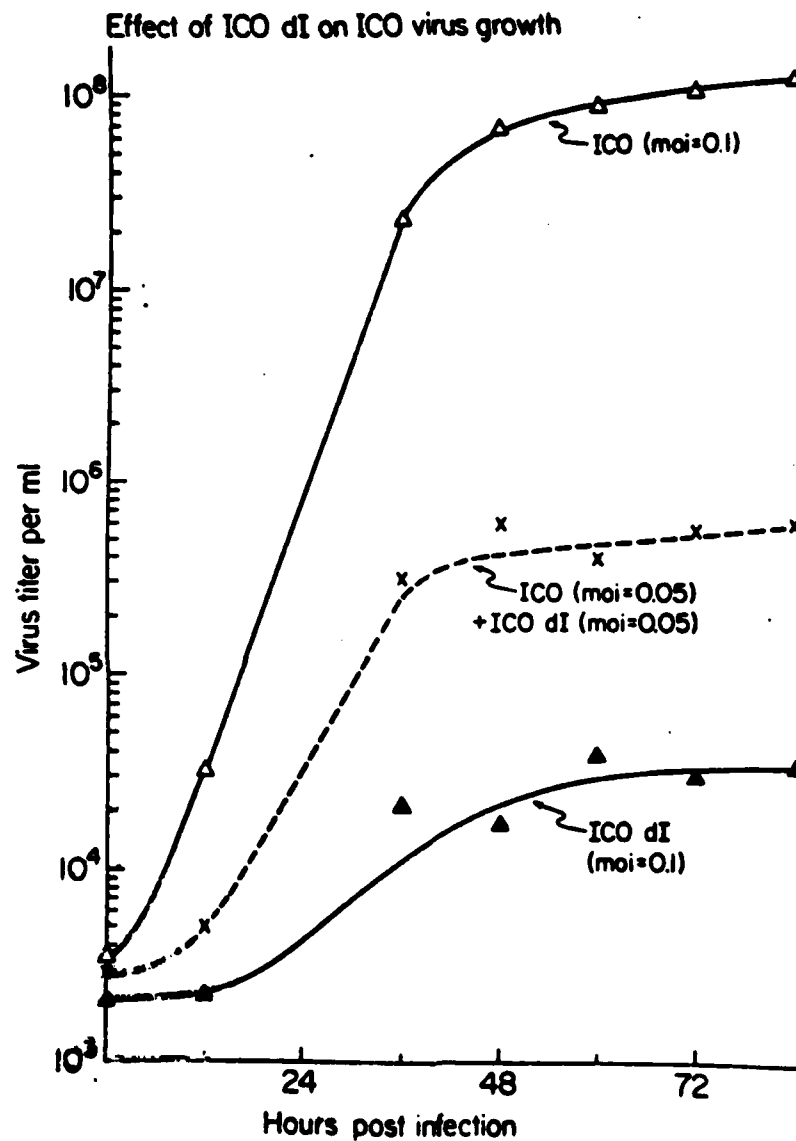
Figure 7

Vero cell monolayers were infected with 2nd passage ICO virus (MOI = 0.1), or 5th passage ICO dI virus (MOI = 0.1), or an equivalent multiplicity of an equal mixture of both stocks (ICO: MOI = 0.05 + ICO dI: MOI = 0.05). Virus progeny were titered as a function of the infection time courses.

An experiment was then undertaken in which virus yields were determined for heterologous viruses grown in the presence of ICO dI virus stocks. The design was to determine if, as in the case of interference of ICO virus by ICO dI, the replication of the heterologous virus was also reduced. Three heterologous viruses have been analyzed: BUE, PT and SSH. The results are expressed as the relative yield of infectious virus as a function of the concentration of ICO dI virus employed (Fig. 8). The single virus infections (i.e., no ICO dI added) gave from 10^7 to $2x10^8$ pfu/ml. The single ICO dI virus infections gave $4x10$ pfu/ml and were therefore not a significant factor. The results obtained indicate that ICO dI virus did not interfere to a significant extent with the replication of BUE, PT, or SSH viruses.
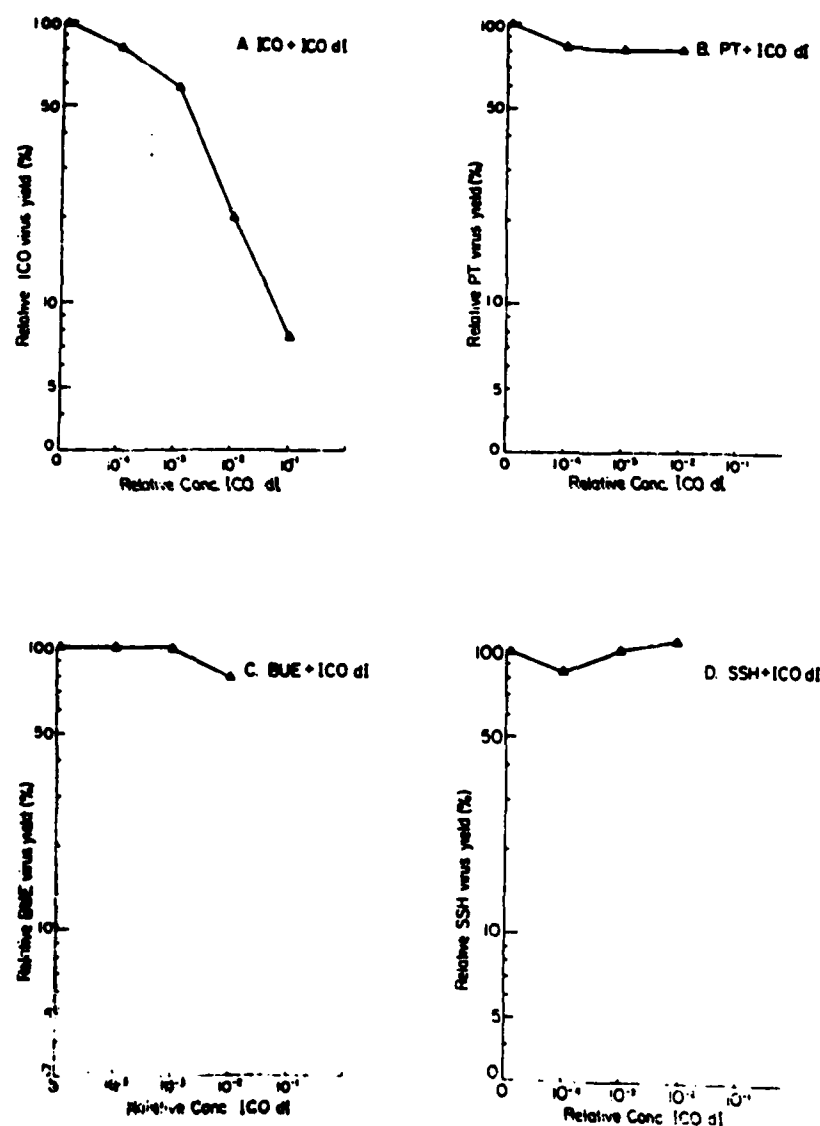

Figure 8

Interference of ICO, BUE, PT and SSH virus by ICO dI stocks. The 60 hr titers of virus obtained in the presence of increasing amounts of ICO dI virus are expressed as the % of the titer of virus grown in the absence of ICO dI virus.

Similar results have been obtained in reciprocal experiments using a stock of PT virus that also exhibits homologous virus interference capabilities.

We conclude from these studies that BUE, ICO and PT viruses are not genetically interactive. Further screening experiments are proposed to examine the capability of ICO, or PT dI virus stocks to interfere with the replication of other phleboviruses.

12). <u>Characterization of the genome composition of ICO dI virus stocks.</u>

Since for a variety of RNA viruses dI particles contain a deleted genome RNA, we initiated a study to characterize the viral RNA present in extracts of ICO dI virus stocks. As shown in Fig. 9 (in particular, the insert) a new band of RNA was found in the dI preparation exhibiting an electrophoretic mobility greater than that of the ICO S RNA. Such a band was not found in RNA extracts of non-interfering ICO virus preparations. By comparison with the mol. wt. estimates of ICO L, M and S viral RNA species (2.8×10^6, 1.8×10^6 and 0.83×10^6, respectively), it was estimated that the dL RNA had a mol. wt. of approximately 0.42×10^6.

Coelectrophoresis of the viral RNA of snowshoe hare (SSH) and Icoraci (ICO)
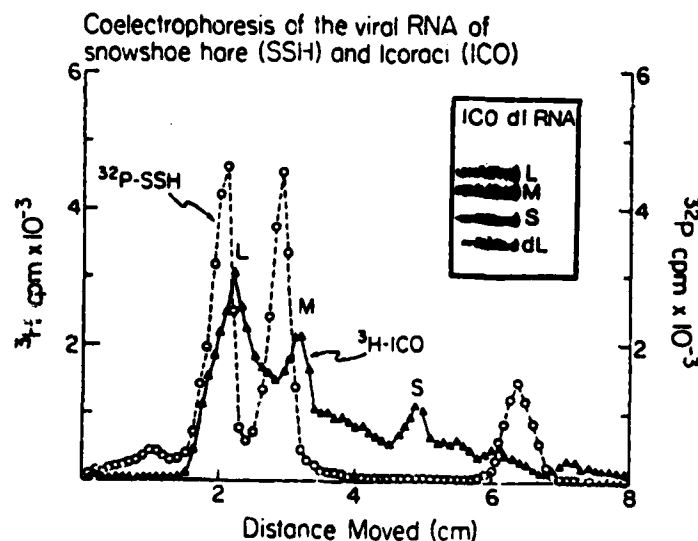
Figure 9

Coelectrophoresis of the viral RNA of ^{32}P-labelled snowshoe hare bunyavirus and ^{3}H ICO RNA. The insert shows an autoradiogram of a ^{32}P-labelled preparation of ICO dI RNA resolved by agarose gel electrophoresis.

15

When the 0.42×10^6 dalton RNA was recovered, fingerprinted and the results compared to the fingerprints of ICO L, M and S viral RNA species, it was deduced that it represented a subgenomic derivative of the ICO L RNA species (see Fig. 10). Similar results have been obtained for the PT dI virus stock, although its L RNA deletion is not as large (data not shown).
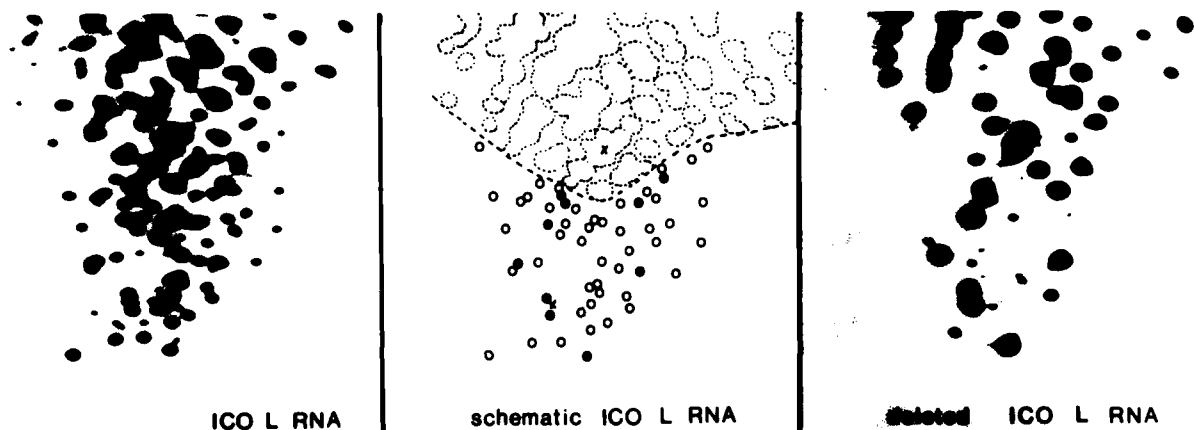

| ICO L RNA | schematic ICO L RNA | deleted ICO L RNA |

Figure 10

Fingerprint analyses of the L RNA of ICO viral RNA (left panel) and the 0.42×10^6 dalton ICO RNA ("deleted ICO L RNA", dL). A schematic of the L RNA fingerprint in the center panel is shown with the filled-in circles representing the oligonucleotides of the 0.42×10^6 RNA.

13). Analyses of the 3' end sequences of BUE and PT viral RNA species.

Preliminary 3' end sequence analyses of ^{32}P-pCp end-labelled BUE and PT L, M and S RNA species indicate that the 3' ends of each RNA of each virus is $_{OH}$UUC... (determined as described for SSH virus, Clerx van-Haaster & Bishop, 1980). This trinucleotide is similar to that of Uukuniemi viral RNA species (M. Hewlett, personal communication).

B. Summary of Progress Report.

The research supported by this contract has shown that representative PHL group viruses (Phlebovirus genus) have a tripartite RNA genome consisting of 3 unique RNA species (L, M and S). The L and M RNA species have mol. wts. like those of Bunyavirus and Uukuvirus genus members. Like uukuviruses, the S RNA species of phleboviruses is significantly larger than that of bunyaviruses. Members of all three of the above genera have a major 20-24x10³ dalton nucleocapsid protein N. Our analyses have shown that the size ranges of phlebovirus glycoproteins and their surface arrangement are similar to those of uukuviruses, and unlike that of Bunyavirus genus viruses.

Rift Valley fever virus (recently classified as a phlebovirus) has viral RNA species and the polypeptide sizes like those of the PHL group viruses and unlike those members of of members of the Bunyavirus genus (Rice et al., 1980; Cash et al., 1980).

We have analyzed the genotypes of various RVF and PT virus isolates by oligonucleotide fingerprinting. The results indicate that genetic variation exists for each virus in their respective Old and New world ecological niches.

The biochemical data developed from the research supported by this contract have been, in part, the basis for the recent proposal (and acceptance) of the group as a new genus in the Bunyaviridae, named the Phlebovirus genus (Bishop et al., 1980).

Genetic studies initiated with Punta Toro (PT) virus have shown that high frequency intertypic genetic recombination occurs during certain PT mixed virus infections (ts, or dual wild-type coinfections using alternate isolates of PT virus). Temperature sensitive (ts) mutants of PT virus have been categorized into groups on the basis of their recombination capabilities (Group I has 8 ts mutants; Group II has 5 ts mutants; Group III has 1 ts mutant; 1 ts mutant is unassigned). From analyses of the reassortants generated from dual infections involving prototype PT and an alternate PT strain, PT-ada, it has been demonstrated that the viral S RNA codes for the N polypeptide. In vitro translation by subgenomic polysome mRNA indicates that N is coded by a small mRNA. Some evidence has been obtained which suggests that a non-structural polypeptide, p30, is also coded by a small subgenomic mRNA representing an S cRNA.

So far no reassortment has been detected between PT and BUE, or BUE and ICO viruses either by analyses of the progeny of dual wild-type virus infections, or from dual infections involving PT ts mutants and wild-type BUE virus (neither of which give plaques at 39.8°) and screening for reassortant progeny at the non-permissive temperature. These results suggest that as in the case of bunyaviruses the gene pools of phleboviruses may be limited.

In addition to the radioimmune assays reported previously with KAR virus to relate the genetic capabilities of phleboviruses with their antigenic similarities, we have developed a system to screen for genetic interaction between phleboviruses based on heterologous virus interference assays. We propose to use this procedure to screen for heterologous virus interference before continuing with genetic recombination analyses.

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